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Facilitation of Human Osteoblast Apoptosis by Sulindac and Indomethacin Under Hypoxic Injury

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ABSTRACT

Hypoxic–ischemia injury occurs after trauma causes consequential bone necrosis. Non-steroid anti-inflammatory drugs (NSAIDs) are frequently used in orthopedic clinics for pain relief. However, the underlying mechanism and outcome for usage of NSAIDs is poorly understood. To investigate the damage and loss of osteoblast function in hypoxia, two hypoxia mimetics, cobalt chloride ($CoCl_2$) and desferrioxamine (DFO), were used to create an in vitro hypoxic microenvironment. The cell damage was observed by decreases of cell viability and increases in cyclooxygenase-2 and cleaved poly(ADP-ribose) polymerase (PARP). Cell apoptosis was confirmed by WST-1 cytotoxic assays and flow cytometry. The functional expression of osteoblast in alkaline phosphatase (ALP) activity was significantly decreased by $CoCl_2$ and inhibited when treated with DFO. To simulate the use of NSAID after hypoxic injury, four types of anti-inflammatory drugs, sulindac sulfide (SUL), indomethacin (IND), aspirin (Asp), and sodium salicylate (NaS), were applied to osteoblasts after 1 h of hypoxia mimetic treatment. SUL and IND further enhanced cell death after hypoxia. ALP activity was totally abolished in hypoxic osteoblasts under IND treatment. Facilitation of osteoblast apoptosis occurred regardless of IND dosage under hypoxic conditions. To investigate osteoblast in vivo, local hypoxia was created by fracture of tibia and then treated the injured mice with IND by oral feeding. IND-induced osteoblast apoptosis was confirmed by positive staining of TUNEL assay in fractured mice. Significant delay of fracture healing in bone tissue was also observed with the treatment of IND. These results provide information pertaining to choosing appropriate anti-inflammatory drugs for orthopedic patients. J. Cell. Biochem. 113: 148–155, 2012.

KEY WORDS: HYPOXIA; OSTEOBLAST; APOPTOSIS; NSAID; ASPIRIN

The human skeleton provides mechanical support for weight bearing and limb movements. Hypoxic–ischemia injury of bone tissues usually occurs after trauma and causes the loss of osteoblast function through the resulting bone necrosis. The microenvironment in the intra-trabecular structure has lower oxygen content as compared to other tissues in the human body [Brighton and Krebs, 1972; Harrison et al., 2002]. However, bone tissue still requires nutrients that are supplied by blood vessels, which are usually blocked as a result of injury in fracture. Abrupt changes in oxygen availability within the periodontium have been suggested to play a regulatory role in alveolar bone remodeling during tooth movement; arguably, the regulation is similar to that seen in bone growth or in the healing of a fracture [Tuncay et al., 1994]. Bone regeneration after fracture involves the intricate cascade of transcriptional events that combine processes observed during embryonic skeletal development and organismal responses to both acute injury and regional ischemia.

In fractured bone, the hypoxia-inducible factor (HIF) signaling pathway will be induced when circulation to a fractured region is blocked [Towler, 2007]. HIF-1α, a heterodimer that functions as a

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transcription factor, is tightly regulated at the protein level under normoxic conditions through ubiquitin-mediated degradation and is stabilized and activated under conditions of hypoxia [Komatsu and Hadjiargyrou, 2004]. HIF-1α is activated during bone repair, and can be manipulated genetically and pharmacologically to augment skeletal healing. However, the effect of hypoxia in osteoblasts is still debated. Local increases in HIF-1α are found to contribute to bone formation in mouse fracture models [Wan et al., 2008]. The long-term effect of hypoxia in osteoblasts is still unknown.

The inflammatory reaction in fractured bone results in severe pain. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in clinics to relieve pain [Vuolteenaho et al., 2008]. However, inhibition of prostaglandin production via the use of NSAIDs and specific inhibition of COX-2 have been shown to inhibit fracture repair [Boursinos et al., 2009]. Thus, the purpose of this study is to understand the characteristic of hypoxia-induced osteoblast damage and the influence of NSAIDs on the sequential mechanism of apoptosis in osteoblasts. The molecular signals for hypoxia, COX2, cell survival, and their functional consequence in bone deposition are assessed in human osteoblasts subjected to cobalt chloride ($CoCl_2$) or desferrioxamine (DFO) for mimic hypoxic conditions in vitro. The damage of osteoblasts with NDAIDs treatments are further confirmed by creating an impact fracture on tibia bone in mice. The findings in the current study have provided meaningful information to contribute to the clinical therapeutic strategies of orthopedic application.

METHODS

CELL CULTURE

Human osteoblast-like cells (MG-63, osteosarcoma cell line, ATCC) were cultured to confluency on 6-cm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin–streptomycin (Invitrogen). Cells were maintained in a humidified incubator at 37°C with 5% CO_2 as previously described [Wu et al., 2006]. Other human osteoblast-like cells (hFOB, ATCC) were also used to confirm the general phenomenon of hypoxia effects on human bone cells. The hFOB were cultured in DMEM-F12K (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin–streptomycin (Invitrogen). Cells were transferred to a humidified incubator at 37°C with 5% CO_2 during the drug treatment.

IN VITRO HYPOXIA MODEL

The ischemic injury model of osteoblasts was carried out by treatment with $CoCl_2$ (Sigma–Aldrich), DFO (Sigma–Aldrich), or reducing the concentration of oxygen in the hypoxia incubator (Autoflow 4950, NuAire Inc.). Both the $CoCl_2$ and DFO were freshly dissolved in autoclaved distilled water and used at concentrations of 100 μM for $CoCl_2$ and 20 μM for DFO to mimic the hypoxic state in vitro. The 2% hypoxic condition was created by mixing 5% CO_2 and replacing oxygen with N_2 in hypoxia incubator. The O_2 sensor was built into the system for indication and controlled the release of N_2. As the cells reached 80% confluency, hypoxic conditions were induced by rinsing the cells with phosphate-buffered saline (PBS) and then incubating with medium containing hypoxic drug supplements or transferring cell into hypoxia incubator at different time points.

ANTI-INFLAMMATORY DRUG TREATMENT

Pharmacological reagents were used to test the anti-inflammatory drugs (AIDs) in hypoxic osteoblasts. Sulindac sulfide (SUL) and indomethacin (IND), purchased from Calbiochem, were dissolved in ethanol. Aspirin (Asp) and sodium salicylate (NaS), purchased from Sigma–Aldrich, were dissolved in ethanol and distilled water, separately. The AIDs were added to the hypoxic culture medium after 1 h of hypoxia treatment.

CELL VIABILITY ASSAY

Cytotoxic activity was assessed using WST-1 kit (Roche) to determine the number of viable osteoblasts remaining after treatment with hypoxic reagents. Cells, grown in a 96-well tissue culture plate for overnight and then treated with hypoxic reagents, were incubated with the WST-1 reagent for 2 h before the indicated time pinots. The stable tetrazolium salt of WST-1 is cleaved by a complex cellular mechanism at the cell surface in viable osteoblasts. The amount of formazan dye formed directly correlates to the number of metabolically active cells. The quantitative measurements of colorimetric assays for the absorbance were read at 450 nm with a scanning multi-well ELISA reader.

Cell apoptosis was further assessed using flow cytometry by positive staining with Annexin V and negative staining with propidium iodide (PI) as described previously [Wu et al., 2007]. Human osteoblasts incubated with hypoxia medium for different time periods were re-suspended and incubated with fluorescein isothiocyanate (FITC)-labeled Annexin V antibody and PI (BD Pharmingen) in the dark at room temperature for 30 min. The labeled cells were measured by flow cytometry (FACScan, BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Percentages of cells with positive staining for Annexin V and negative staining for PI were calculated for identification of apoptotic cells.

WESTERN BLOTTING

The osteoblasts were rinsed twice with cold PBS and then lysed with RIPA buffer containing protease inhibitors [Wu et al., 2006]. Cell lysate supernatants containing 20 μg of total protein were transferred to nitrocellulose membranes (Bio-Rad Co.) following SDS–PAGE on 10% cross-linked gels. The membranes were blocked by 5% dry milk in TBS with 0.1% Tween-20 for 90 min. For HIF-1α detection, membranes were incubated in an anti-HIF-1α antibody (1:1,000 dilutions; Novus) overnight at 4°C. A rabbit poly(ADP-ribose) polymerase (PARP) antibody (1:1,000 dilutions; Cell Signaling Technology) was used to detect full-length PARP (116 kDa) and cleaved PARP (carboxyl-terminal catalytic fragment, 89 kDa). Expression of cyclooxygenase-2 (COX-2) was detected by rabbit polyclonal antibody (1:1,000 dilutions; Millipore). Bound primary antibodies were detected using appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology) and an ECL detection system (Amersham, Uppsala).

FUNCTIONAL TEST OF ALKALINE PHOSPHATASE

Alkaline phosphatase (ALP) activates bone deposition and can be detected in healthy osteoblasts. The functional activity of osteoblasts under hypoxic conditions was observed by treatment with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). Human osteoblasts (hFOB) were fixed by 4% paraformaldehyde (Sigma) and then stained using an ALP kit (Sigma) in accordance with the instructions provided by the manufacturer. To quantify the production of ALP, the soluble substrate (*p*-nitrophenyl phosphate, pNPP) for the detection of ALP activity in colorimetric assays of enzyme activity was used, and the absorbance was read at 405 nm.

IN VIVO HYPOXIA MODEL

The 8-week-old female B6 mice were used to create local fracture and study the damage of osteoblasts with IND treatment during ischemic injury in vivo. The experimental procedures for fracture model in mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at NCKU. Briefly, the animal fracture model was created by inserting a steel pin (0.25-mm diameter) into the left tibia and then applying a vertical impact force (5.15×10^{-1} N) by using a free-dropping impacted-injury simulator on the middle shaft of tibia in the anesthetized mice. The X-ray images were performed to confirm the complete fracture of tibia and to prevent misalignment after inserting steel pin during fracture healing. After the fracture injury, the IND was oral fed daily (1.5 mg/kg/day) by using oral feeding needle (0.9 mm × L50 mm, Nazme, Japan). The apoptosis of osteoblasts in fractured tissue were labeled by tissue fixation, decalcification, dehydration, paraffin embedding, section, and then staining for DNA strand breaks in individual cells by using in situ cell death detection kits (Roche) according to the recommendation protocols. The TUNEL positive osteoblasts were visualized with immunocomplexed of POD substrate reaction in injured tissue after 2 days, 1 week, and 4 weeks of bone fracture. The osteoblasts located at both outer and inner boundary of cortical bone were identified and measured the percentage of positive stained osteoblasts from three independent experiments.

STATISTICAL ANALYSIS

Data reported are means ± standard errors from three independent experiments for each time point and each treatment group. The differences between the control and treatment groups were analyzed by one-way ANOVA and Bonferroni tests for multiple comparisons, with a P-value <0.05 accepted as statistically significant.

RESULTS

HYPOXIA-INDUCED OSTEOBLASTS DAMAGE IN VITRO

The hypoxic conditions induced morphological changes at 24 h when MG-63 cells were subjected to $CoCl_2$ or DFO (Supplementary Fig. 1). The decrease in WST-1 reading indicated the cytotoxicity with treatment of hypoxia mimetics (Fig. 1A). Cell death was further assessed by AnnexinV/PI staining and confirmed using flow cytometry. Decreases in viable cell numbers were observed following both $CoCl_2$ and DFO treatments (Fig. 1B). Treatment with hypoxia mimetics increased cell apoptosis as shown by the shift in the cell population to Annexin V-positive and PI-negative staining following $CoCl_2$ treatment or PI-positive staining following DFO treatment.

To explore intracellular signaling in the early stages of hypoxia, the MG-63 cells were treated with hypoxia reagents at different time points. The indicators of programmed cell death by cleavage of PARP were significantly increased at 24 h for $CoCl_2$ and 48 h for DFO (Fig. 1C). These results suggested that treatment with hypoxia mimetics caused osteoblast damage in MG-63 cells. Both $CoCl_2$ and DFO slightly induced expression of HIF-1α (Fig. 1D). The expression level of COX-2 protein was significantly increased by as early as 4 h for both hypoxia reagent treatments (Fig. 1E). To assess the hypoxia-induced osteoblast injury phenomenon, a different human osteoblast cell line (hFOB) was used, and similar results were obtained (Supplementary Fig. 2). The hypoxia-induced cell damage was further confirmed by increased cleaved PARP when MG-63 cells were cultured under 2% oxygen in the hypoxia chamber for 24 and 48 h (Supplementary Fig. 3).

LOSS OF ALP FUNCTION IN HYPOXIA-INJURED OSTEOBLASTS

The histological assessment of ALP was performed to assay for osteoblast function. The synthetic function of bone matrix in osteoblasts in vitro was detected using the BCIP/NBT reaction, which results in blue coloration. In normal conditions, the osteoblasts can produce ALP and increase staining intensity during prolonged cell culture (compare the 72 h to 24 h in the control group; Fig. 2A). The phase images demonstrated that the loss of ALP function was accompanied by morphology changes in hypoxia-injured hFOB cells, especially following $CoCl_2$ treatment (Fig. 2B). The soluble substrate of ALP was further used to quantify the ALP production and measured by colorimetric assay. The $CoCl_2$ caused a reduction in ALP function by as early as 24 h in hFOB cells (Fig. 2C). DFO treatment also prohibited ALP production compared to the control group at 72 h.

NSAIDs ENHANCE CELL DEATH IN HYPOXIC OSTEOBLASTS

We assumed that the hypoxia-induced cell damage was triggered by COX-2 activation and might cause several inflammatory responses. However, bone healing also requires appropriate COX-2 expression. Treatment with NSAIDs can affect the outcome of fracture for orthopedic patients. Therefore, we were interested in treating hypoxic osteoblast with commonly used NSAIDs. Several AIDs, including SUL (160 μM), IND (800 μM), Asp (1,000 μM), and NaS (1,000 μM), were applied to hypoxic osteoblasts 1 h after hypoxia occurred to mimic the treatment strategies in trauma. The treatment of SUL and IND caused further cell damage with significant decreases of WST-1 cytotoxic activities (Fig. 3A). The ALP activities were abolished, as measured by colorimetric assay, and significant cell detachment was observed in hypoxic osteoblasts following treatment with SUL or IND (Fig. 3B). The cleavage of PARP confirmed the increase in cell apoptosis in hypoxic osteoblasts treated with SUL or IND under hypoxic conditions (Fig. 3C). Interestingly, COX-2 expression was also increased following SUL or IND treatment compared to hypoxic osteoblasts treated with vehicle

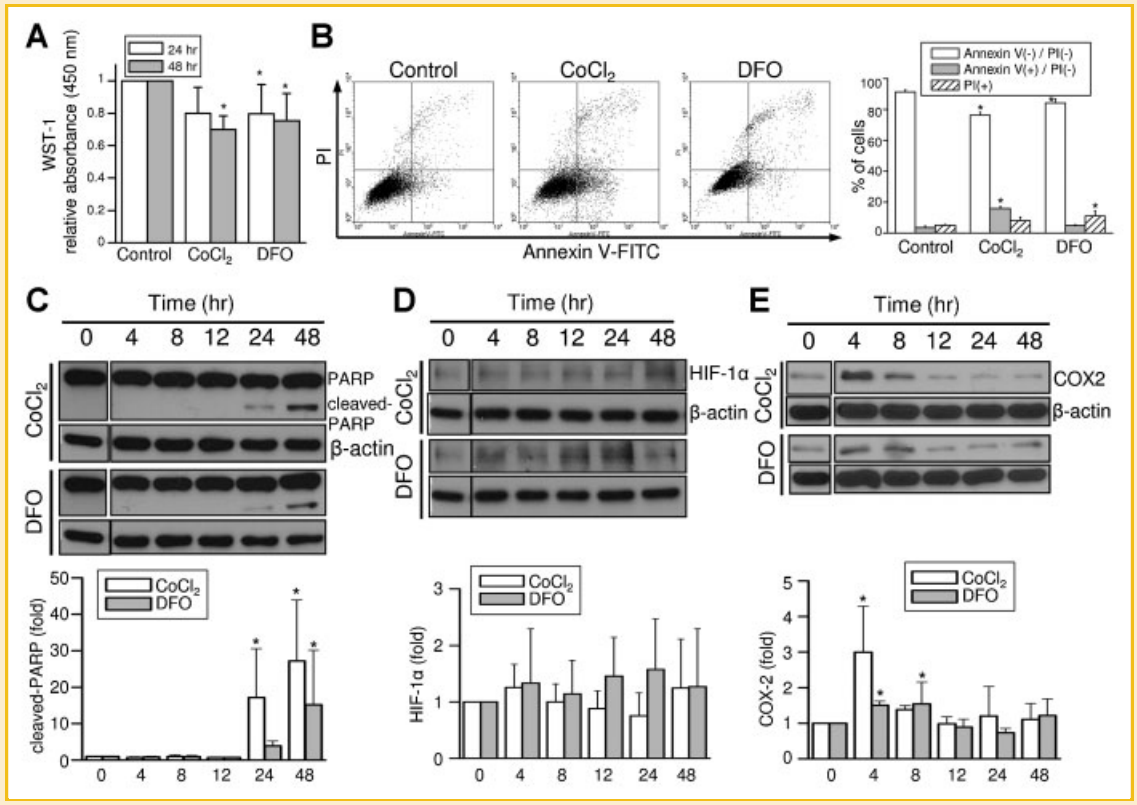

Fig. 1. A decrease of WST-1 reading indicated the cytotoxic activity (A) and confirmed the cell apoptosis using Annexin V/PI staining with flow cytometry (B) in human hFOB osteoblasts treated with hypoxic reagents. Increasing the cleavage of PARP also indicated cell damage after 24 h of hypoxia (C). Both $CoCl_2$ and DFO induced a hypoxic microenvironment in vitro and resulted in slightly increased HIF-1α expression in MG-63 cells (D). The application of hypoxic reagents induced transient induction of COX2 protein expression between 4 and 8 h (E). *Significant difference ($P < 0.05$) from control.

control ($CoCl_2$ or DFO only; Fig. 3D). No significant change in cell death was observed with NSAID treatment under normoxia conditions (without treatment of hypoxic reagents under 20% of oxygen; Supplementary Fig. 4). Conversely, Asp showed an intriguing outcome in that it ameliorated the hypoxic injury. Decreases in cleaved PARP and COX-2 expression suggest that osteoblasts were protected from hypoxia-induced cell damage (Fig. 3C,D). The colorimetric readings showed no further decrease in ALP activity following treatment with Asp and NaS under hypoxic injury (Fig. 3B).

DOSAGE INDEPENDENCE OF INDOMETHACIN-ENHANCED OSTEOBLASTS DEATH UNDER HYPOXIA

IND is commonly used in orthopedic clinics due to its effectiveness in pain relief. However, the IND-induced increase in cell apoptosis and death in hypoxic osteoblast was confirmed using AnnexinV/PI flow cytometry (Fig. 4A). An 800 μM dosage, as previously described, was relatively higher than the physiological concentration (75–100 mg/day for a patient weighing 50 kg). We treated hypoxic injured osteoblasts with IND at concentrations of 800, 400, 200, and 100 μM. In the presence of hypoxic conditions, even the lowest dose (100 μM IND) enhanced cell damage as shown in phase images (Fig. 4B) and by increased cleaved-PARP levels (Fig. 4C). These results suggest that cell damage was facilitated and functional restoration was inhibited when IND was applied to osteoblasts in the hypoxic injury model.

INDUCTION OF OSTEOBLASTS DEATH WITH INDOMETHACIN TREATMENT IN FRACTURED MICE

The osteoblasts are mainly located at the boarder of cortical bone in long axial skeleton. The uninjured bone showed an intact tibia shaft and clear soft tissue structures in healthy mice (Fig. 5A). The in situ TUNEL section showed negative staining in osteoblast cells. The fracture increased acute positive staining of osteoblast at 2 days, peak at 1 week, and reduced during the healing processes at 4 weeks after injury (Fig. 5B, upper panels). However, the treatment of IND (1.5 mg/kg) enhanced the amount of TUNEL positive osteoblasts in different time points and reduced the regeneration of cortical bone structure after 4 weeks of injury (Fig. 5B, lower panel). The significant increases of cell apoptosis in IND-treated mice were confirmed by counting the percentage of TUNEL positive osteoblast in vivo (Fig. 5C).

DISCUSSION

Our results demonstrate successful models for investigating the ischemic injury of bone tissue both in vitro and in vivo. HIF-1α was induced by both $CoCl_2$ and DFO in human osteoblasts (MG-63 and

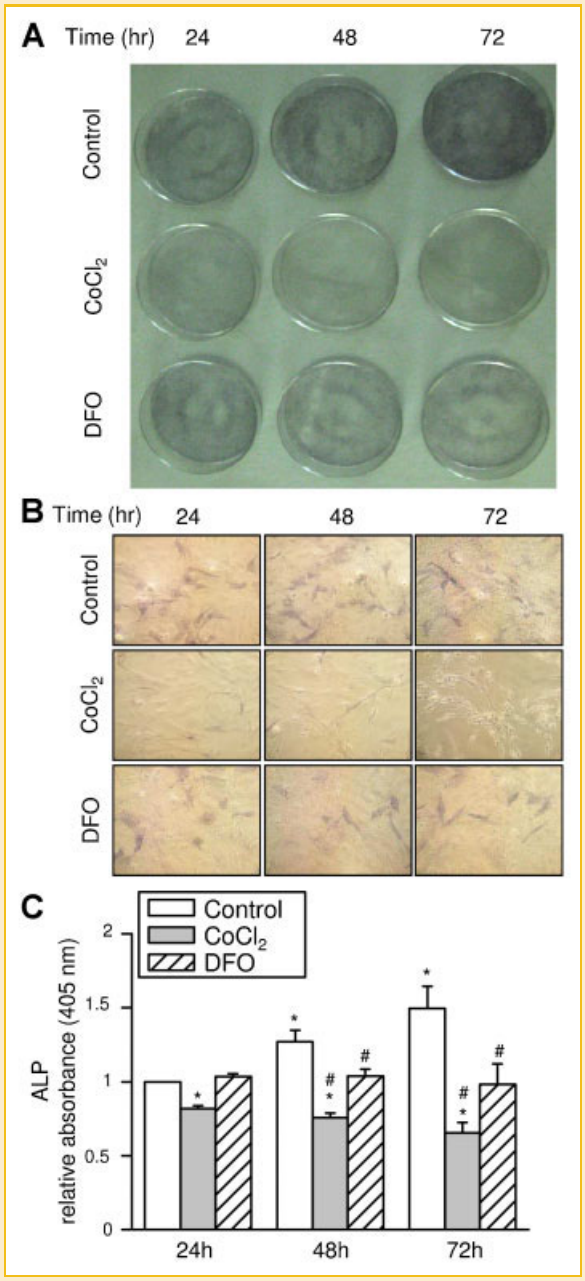

Fig. 2. Hypoxia led to a loss of ALP activity in osteoblasts. Decreased ALP production was observed based on the reduction of blue BCIP/NBT staining (A). Morphological changes in phase images were closely correlated to the loss of ALP function (B). The soluble substrate of ALP was further used to quantify ALP production and measured by colorimetric assay at 405 nm absorbance (C). *Significant difference ($P < 0.05$) from control at 24 h. #Significant difference ($P < 0.05$) from control at relative time point.

hFOB cells). Prostaglandin is an important molecule for bone repair and inflammatory reaction. Changes in prostaglandin synthesis and concentration have been shown to correlate with the regeneration of trabecular bone for accelerating fracture healing [Weinreb et al., 1997]. Osteoblastic cells increased prostaglandin E_2 (PGE_2) release and the expression of its receptor EP_1 in response to hypoxia (2% oxygen) [Lee et al., 2007]. Treatment with another hypoxic reagent, dimethyloxaloglycine (DMOG), also increased EP_1 protein expression and suggested that a relationship exists between prostaglandin synthesis and HIF signaling [Genetos et al., 2009]. HIF-1α was not induced in osteoblasts until the hypoxic reagent was added to the in vitro culture environment (Fig. 1D). The current study concludes that osteoblasts are promptly damaged under hypoxic conditions, as indicated by PARP cleavage, cell viability, and Annexin V/PI staining (Fig. 1). The positive staining of TUNEL tissue section confirmed the apoptosis of osteoblasts in fractured bones (Fig. 5B).

Signal transduction and gene expression profiles are essential for distinguishing between normal and injured bone tissue [Su et al., 2011]. Several molecules are involved in bone remodeling via modulating the concentration or activity of transforming growth factor beta 1 [Sakai et al., 1998], COX-2 [Chen et al., 2000], collagen types I (COL1), collagenase-3, EGR-1, osteocalcin [Rodan and Noda, 1991], and osteopontin (OPN) [You et al., 2001; Wu et al., 2006] in bone cells. Signal transduction-induced bone remodeling can be initiated by increasing the intracellular calcium concentration [Chen et al., 2000] as a result of opening the stretch-activated channel; this leads to the release of nitric oxide [McAllister and Frangos, 1999]. Signal transduction involves the sequential activation (via a phosphorylation cascade) of various intracellular proteins, including mitogen-activated protein kinases (MAPKs) [Wu et al., 2006; Lee et al., 2008], phosphoinositide 3-kinase (PI3k)/Akt [Danciu et al., 2003; Lee et al., 2010], and protein kinases B and C [Biggs et al., 1999; Geng et al., 2001]. As a result, these signals can activate the transcription factors that modulate the expression of genes that regulate different physiological functions of bone metabolism. Examples of these transcription factors include activator protein-1 transcription factor [Peverali et al., 2001], bone-specific transcriptional regulator [Ziros et al., 2002], and NF-κB [Granet et al., 2001]. For gene regulation and indication of bone function, the decrease of ALP activities was observed after hypoxia. In addition to ALP activities, the function of osteoblasts in bone matrix metabolism can be evaluated by expression levels of COL1 (which is important for ECM synthesis) and OPN (which regulates calcium deposition) by quantifying gene expression using real-time PCR [Wu et al., 2006]. The histological assessment of ALP can directly reflect the functional outcome of tissues and is observed by the eye (Fig. 2A) and quantified by colorimetric assay (Fig. 2C).

Hypoxia may induce inflammatory reactions after bone fracture. Several cytokines in hypoxic tissue could also trigger the HIF-1α signaling, such as IL-1β and TNF-α [Haddad and Harb, 2005]. The signal cascade is initiated by receptor-mediates mechanisms. Under the normoxic condition, the cytokine-induced HIF signaling is inhibited by HIF ubiquitination. In hypoxic tissue, the cytokines could play additional effect to up-regulate HIF-1α. On the other hand, NSAIDs could affect expression levels of HIF-1α and/or the von Hippel Lindau tumor suppressor (VHL) [Jones et al., 2002]. Under hypoxic condition, VHL expression levels are suppressed leading to HIF-1α accumulation, VEGF/Flt-1 expression, and angiogenesis in endothelial cells. In the presence of NSAIDs, VHL is up-regulated leading to increased ubiquitination and degradation of HIF-1α, causing reduced VEGF/Flt-1 expression and inhibition of hypoxia-induced angiogenesis. The AIDs used in the current study are related to several commercial pharmacological drugs. The SUL

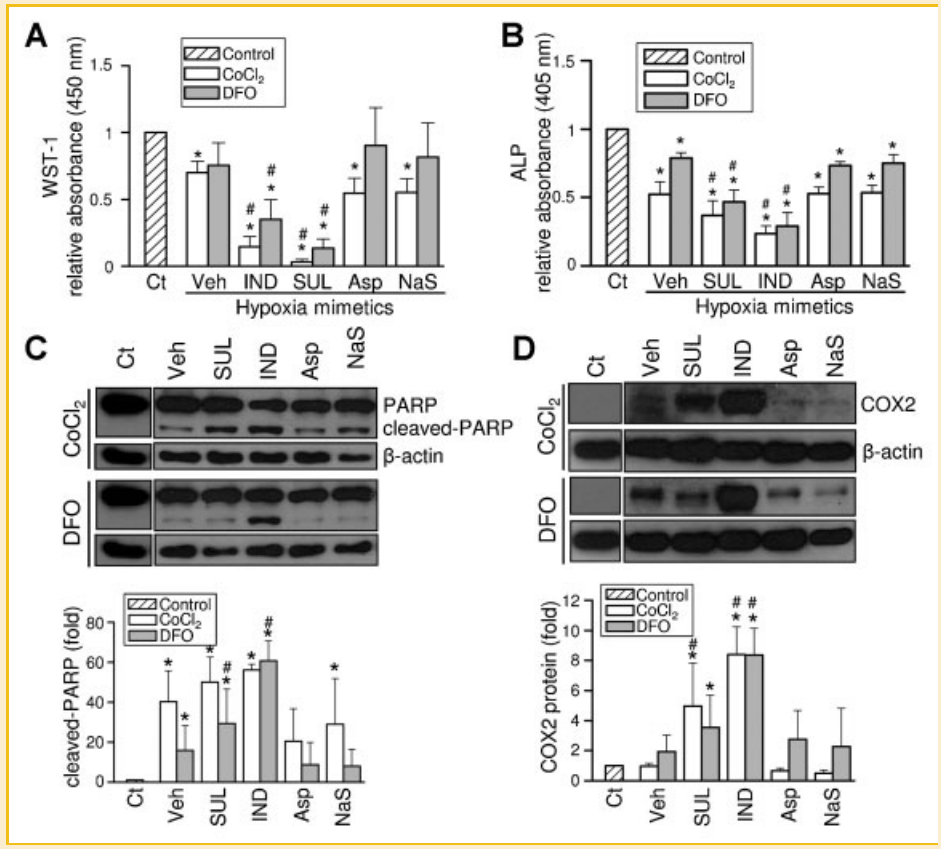

Fig. 3. After 1 h of hypoxia, the effect of NSAID application in damaged osteoblasts was assessed by adding SUL, IND, Asp, or NaS. Facilitation of cell death following treatment with SUL or IND were shown by further decreased WST-1 activities (A), abolishment of ALP activities (B), increase of PARP cleavage (C), and higher COX-2 protein expressions (D). The results suggest that cell damage was enhanced and ALP function was inhibited when SUL and IND were applied to hypoxic osteoblasts. *Significant difference ($P < 0.05$) from control. #Significant difference ($P < 0.05$) from the relative hypoxic group.

(sulindac) is similar in chemical structure to Clinoril. IND is commonly recommended for pain relief in orthopedic clinics. Before the discovery of NSAIDs, Asp and NaS were also popular for analgesics and anti-pyretics [Warner and Mitchell, 2002]. Although SUL and IND caused endothelial cell apoptosis under normoxic conditions [Liou et al., 2008], the application of all AIDs on osteoblasts under normoxic conditions did not cause significant cell damage as shown by trypan blue staining (Supplementary Fig. 4).

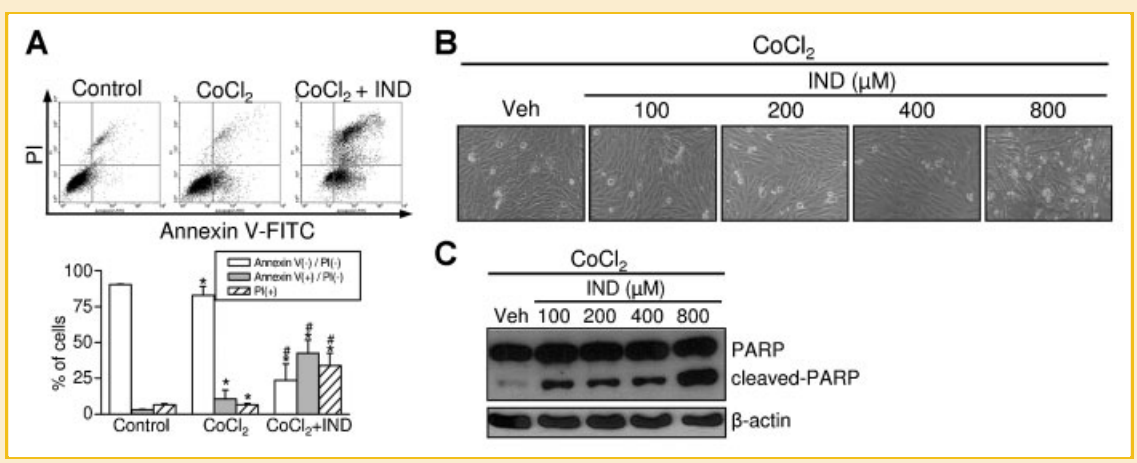

Fig. 4. IND enhanced cell damage by increasing both apoptosis (Annexin V positive, PI negative) and cell death (PI positive) in hypoxic-injured osteoblasts (A). Cell damage occurred at the lowest dosage of 100 μM, as assessed by cell morphology changes (B) and increased PARP cleavage (C). *Significant difference ($P < 0.05$) from control. #Significant difference ($P < 0.05$) from the hypoxic group without treatment of IND.

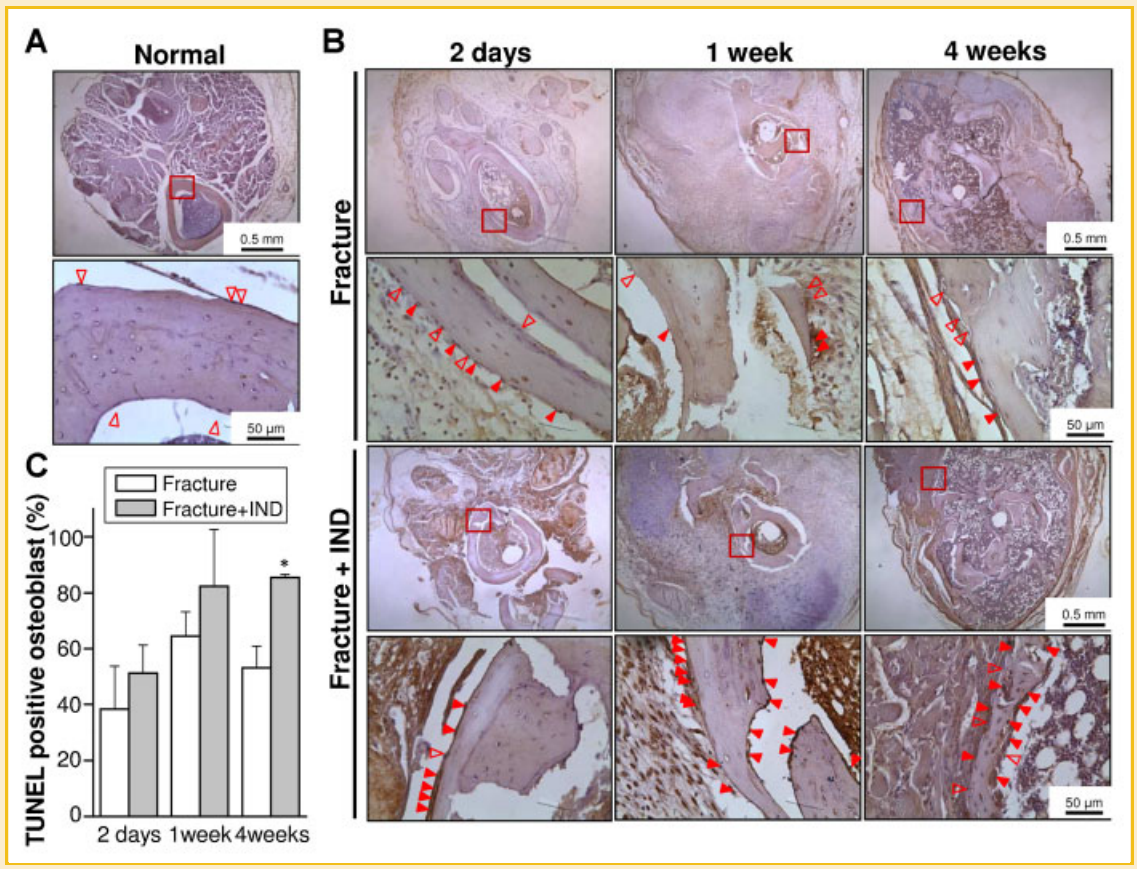

Fig. 5. In healthy mice, the intact tissue showed a negative staining of TUNEL in histological section of middle leg (A). The impact fracture of tibia caused transient increase of cell apoptosis by in situ TNUEL staining (B, upper panel). The healing of fractured bone was observed after 4 weeks of injury. The treatments of IND enhanced the positive stained osteoblasts and delay the healing of cortical bone in fractured tissue (B, lower panel). The peak of osteoblast apoptosis were measured after 1 week of fracture by calculating the percentage of TUNEL positive osteoblast near the border of cortical bone (C). The IND treatment significantly increased the number of cell death among different time points of fracture. *Significant difference ($P < 0.05$) from the fracture group without treatment of IND.

This indicated that cell toxicity was not evident at the given concentrations in osteoblasts. Pain relief and bone healing are both important in patients with bone fractures. IND was suggested due to its effective pain relief qualities in clinics. However, the results obtained in the current study indicate that in hypoxic conditions, even the lowest dose of IND (100 μM) can trigger further cell damage (Fig. 4B,C). The animal model for IND treatments in fracture mice showed a significant increase of osteoblast apoptosis and delay of bone healing in injured tissue (Fig. 5). The clinical use of IND is within the dosage range of our current data and suggests that IND should be used cautiously in fracture patients. To our surprise, a high dose of Asp did not increase osteoblast apoptosis following treatment with both hypoxic reagents. Instead, Asp rescued the osteoblasts from hypoxic damage. COX-2 expression was decreased following treatment with Asp, suggesting that inflammatory reactions in hypoxic microenvironment were inhibited (Fig. 3D). However, the detailed mechanism between COX-2 expression and PGE_2 production in response to bone formation remains unknown.

The complicated microenvironment of fractured bones limits the understanding of molecular mechanisms during injury and healing processes. Although the microenvironment in bone has lower oxygen level (with the range from 2% to 5% of oxygen), the blockage of nutrition supply still resulted an induction of HIF signals in fracture tissue. Revealing the molecular mechanisms can improve the therapeutic strategies for enhancing bone regeneration and improving clinical outcomes. The in vitro hypoxia model can provide a fast drug-screening system for osteoblast regeneration, as presented in the current study. The underline molecular mechanism with the correlation to fracture healing could be investigated by using current model demonstrated in current study. NSAIDs may also effect the proliferation and differentiation of osteoblast in vivo. Although we did not observe the delay of cell proliferation when treating NSAIDs in both MG-63 and hFOB cells under normoxic conditions, the application of current in vitro model for primary osteoblast culture might able to reveal the effects on osteoblast differentiation. Furthermore, animal studies should be performed to confirm these in vitro findings and to illustrate the optimized dosages for AIDs in vivo. In summary, we found that Asp might be better at preventing hypoxia-induced cell damage, whereas other drugs (IND and SUL) facilitated cell death. These findings are important for improving the clinical therapy strategies for orthopedic applications.

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